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The purpose of this project is to determine the role of FGF receptor 1 in reactive stroma during prostate tumorigenesis. We are using a novel approach to target transgene expression specifically to the reactive stroma of experimental prostate cancer. We are placing an inducible Cre recombinase into the FAP gene locus to target expression to reactive stroma. We will cross this mouse with *Fgfr1*^{flox} mice (*LoxP* sites flanking FGF receptor 1 alleles). These mice will be crossed with TRAMP mice (prostate cancer model). Induced expression of Cre at sites of reactive stroma generated in the cancer foci will function to excise the FGF receptor 1 alleles and create a conditional knockout mouse. Progression of tumorigenesis in this line of knockout mice will be compared to heterozygous and wild type controls. We have acquired all clones and reagents and have completed rederiving the *Fgfr1*^{flox} mice by embryo transfer. We have nearly completed generating and verifying the constructs necessary to target Cre and the five generations of breeding necessary to obtain the desired genetic background of mice. This study will pinpoint the role of FGF receptor in prostate cancer progression and will help in designing a targeted therapeutic to cancer reactive stroma.

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Introduction:

Stromal responses to altered homeostasis are a generic hallmark of mammalian tissue biology. This response occurs in the stromal compartment of cells, which generally includes fibroblasts, immune components, nerves, and vasculature. The stromal response seems to be a general and predictable response that is observed at sites of wound healing, fibrosis, cardiovascular disease, and in cancer (1). In the predominant epithelial cancers, such as breast cancer, prostate cancer, and colon cancer, reactive stroma evolves at the site of carcinoma initiation and evolves during cancer progression (1, 2). Less well understood, is a clear understanding of the key cell types and the specific mechanisms that regulate reactive stroma formation. Our studies have specifically characterized reactive stroma during the initiation and progression of prostate cancer (3). We have also developed the DRS (differential reactive stroma) xenograft model to assess the role of reactive stroma on tumorigenesis (4-6). Although current evidence indicates that reactive stroma is key for early cancer development and may be involved in metastatic spread, the specific mechanisms of how reactive stroma promotes tumorigenesis are not understood. Our data to date indicates that a transforming growth factor beta (TGF-b) and fibroblast growth factor-2 (FGF-2) feedback loop may be key in this process (5). In order to more completely address specific mechanisms it is now necessary to develop a transgenic mouse model with targeted gene expression in the reactive stroma compartment fibroblasts. Although it is possible to target transgene expression generally to fibroblasts (7), it is not yet possible to target specifically to fibroblasts in a tissue-specific manner or selectively to cancer reactive stroma. The purpose of our project is to use the fibroblast activation protein (FAP) gene locus to target transgene expression specifically to cancer-associated reactive stroma and to use this technology to assess specific mechanisms of FGF-2 signaling in a mouse model of prostate cancer. To accomplish this, we have proposed three Specific Aims and Tasks that will culminate in a conditional knock out of the FGF receptor 1 gene (cognate receptor for FGF-2) in the reactive stroma tumor microenvironment of the TRAMP mouse model for prostate cancer.

Body:

Task 1 will knock-in DNA encoding the Mifepristone (RU 486) inducible Cre recombinase (CrePR1) into the fibroblast activation protein (FAP) locus.

The purpose of this Task is to create a transgenic mouse that has expression of a Miphepristone inducible Cre recombinase (CrePR1) directed by the native FAP gene promoter and regulatory elements. We have proposed using the FAP locus, since this gene and protein is expressed in fibroblasts specifically at sites of reactive stroma formation and not in adult normal fibroblasts (8, 9). FAP has also shown to be regulated by TGF-β (10), a major regulator of reactive stroma. Accordingly, the construction of this mouse will allow us to regulate, via administration of Miphepristone, the expression of CrePR1 at sites of reactive stroma formation. This should, therefore, allow for

expression of CrePR1 in specifically in prostate cancer reactive stroma, once this mouse has been crossed with the TRAMP mice (Task 3).

There were two strategic options that can be used to construct this mouse. One is to use homologous recombination to knock in the Miphepristone inducible Cre recombinase into the native FAP locus. While this is a viable option, recent data indicates that disruption of the native FAP locus could, itself, alter cancer progression. There are ways around this. One method is to knock into the end of the coding sequence behind an IRES element and hope this does not disrupt expression of the native gene. Another method is to isolate a mouse BAC clone (bacterial artificial chromosome) that contains the entire mouse FAP gene (including 5' upstream regulatory sequences) and knock the CrePR1 into that gene just downstream of the start site. This construct will express CrePR1 under the control of the FAP regulatory sequences, however, will not express additional native FAP. This engineered BAC gene can then be used to construct a transgenic mouse without the labor of screening for homologous recombination in mammalian ES cells. We have chosen this approach since this represents the best method to assure that we do not disrupt the endogenous locus, which would complicate interpretations of our results.

To date, we have successfully acquired and screened a BAC (bacterial artificial chromosome) clone (RP23-161B24) that contains the full-length mouse FAP gene. We have confirmed the clone contains the full FAP gene by restriction digestion. We have completed several subcloning steps and prepared CrePR1 with an attached upstream IRES element and an SV40 driven Neo selectable marker and we have flanked the marker by Flp recombinase sites, in order to excise this segment later. We have verified orientation in this construct and fidelity by sequencing. We have acquired EL250 bacterial cells from Neil Copeland. These cells are temperature sensitive for bacterial recombination and contain an arabinose inducible *flpe* gene. We are currently in the process of modifying the CrePR1 construct to prepare for homologous recombination into the BAC clone using the EL250 cells. Once the EL250 clones that have been selected (Neo resistance), we will use Flp recombinase to excise the whole SV40 neo cassette, leaving the IRES-CrePR1 in the FAP locus intact. Once this has been completed, this construct will be used to make a transgenic mouse using our Core Facility as proposed. We anticipate completing Task 1 and will have the FAP(CrePR1) transgenic mouse in the next 4-7 months.

Task 2, is to cross these mice with a EF-1α / lox stop cassette / FGFR1Δ (dominant negative FGF receptor type I-myc tag) to create a FAP(CrePR1) / lox(stop) FGFR1Δ bigenic animal.

It was our hypothesis that FGF receptor 1 signaling in the reactive stroma fibroblasts/myofibroblast is a key signaling process in the evolution and biology of the reactive stroma tumor microenvironment. If, indeed, reactive stroma is tumor promoting, then loss or inhibition of FGF receptor 1 signaling would be expected to inhibit reactive stroma formation and therefore would inhibit tumorigenesis. Hence, the overall goal of Task 2 was to knock down (inhibit) FGF receptor 1 expression or signaling specifically in the reactive stroma fibroblasts. The original specific steps of Task 2 were two-fold. One, was to construct a transgenic mouse that uses the EF-1α promoter upstream of a lox stop

cassette, which is upstream of a dominant negative FGF receptor one construct (FGFR1Δ). This mouse could then be used to cross with the inducible Cre mouse made in Task 1. At sites of reactive stroma (FAP expression), we would then be able to induce expression of the dominant negative FGF receptor 1, which would inhibit native FGF receptor 1 signaling. We have isolated and acquired clones, which contain the EF-1α promoter, the lox stop cassette and the dominant negative FGFR1.

During the construction of the EF-1α / lox stop cassette / FGFR1Δ construct, we became aware of new data published by a Finnish group (11) characterizing a mouse they engineered with *loxP* sites flanking the native FGFR1 gene, thus creating a floxed FGF receptor 1 mouse (*Fgfr1*^{flox}) in the ICR genetic background. The heterozygous and homozygous mice for the FGFR1floxed alleles are phenotypically identical to wild type (11) hence this mouse is an excellent choice for generating a conditional knockout. Since the goal of task 2 is to create a mouse with an inhibited or loss of FGF receptor 1 signaling, it would clearly be more optimal to use the *Fgfr1*^{flox} mice to generate a conditional knockout line (minus FGFR1) as opposed to inhibiting FGFR1 signaling by expression of FGFR1Δ.

Accordingly, with the report of this new FGFR1 floxed mouse line, we initiated a formal collaboration with Juha Partanen (University of Helsinki, Finland) and have acquired the *Fgfr1*^{flox} mice. As these mice were from a non-approved animal facility outside the United States, the Baylor Transgenic Mouse Facility (TMF) required us to rederive the line via embryo transfer. We completed this in the first 3 months of the project period and the rederived colony is now in our pathogen free TMF. This mouse line is maintained as an ICR outbred strain and we have been breeding it into both the FVB and C57BL/6 backgrounds for the last 8 months. The resulting lines will be suitable for crossing with TRAMP mice (C57BL/6 background) in Task 3. For experimental tumorigenesis, female TRAMP mice (C57BL/6) are crossed with male FVB. The male progeny exhibit rapid prostate tumorigenesis and metastasis.. Our strategy has been to separately cross the *Fgfr1*^{flox} mice into both the FVB and C57BL/6 backgrounds for at least 5 generations, which should theoretically yield a <5% ICR genetic background in each resulting line. We currently have completed 4 generations of crosses in each background. Hence, we anticipate having breeders that are homozygous *Fgfr1*^{flox} (FVB) and homozygous *Fgfr1*^{flox} (C57BL/6) ready by the time Task 1 is completed.

Once the crossing is completed, we will cross a high expression FAP(CrePR1) line (also in the FVB background) (made in Task 1) with the homozygous *Fgfr1*^{flox} (FVB), to create the bigenic FAP(CrePR1)/ *Fgfr1*^{flox} (FVB) mouse line (heterozygous floxed FGFR1 allele). This step will complete Task 2. In addition to the proposed experiments for Task 2, we have also generated a fibroblast cell line from the *Fgfr1*^{flox} mouse line peritoneum to use for testing.

Task 3, is to cross this bigenic animal with TRAMP mice. The TRAMP / FAP(CrePR1) / lox(stop) FGFR1Δ bigenic cross should exhibit RU 486 regulated expression of the dominant negative FGF receptor I transgene in TRAMP reactive stroma.

The TRAMP mouse is in the C57BL/6 background. Rapid tumor development occurs when female TRAMP mice are crossed with male FVB breeders. Accordingly, to fully analyze the effects of FGF receptor 1 signaling in reactive stroma, the first step of

Task 3 will be to cross homozygous TRAMP mouse with the homozygous *Fgfr1*^{flox} (C57BL/6 background) line generated in Task 2. Inbreeding of this line will yield the TRAMP/ *Fgfr1*^{flox} line of mice in the C57BL/6 background and heterozygous for floxed FGFR1 allele and homozygous for TRAMP. The second and final step of Task 3 will be to cross these TRAMP/ *Fgfr1*^{flox} (C57BL/6) mice with the FAP(CrePR1)/ *Fgfr1*^{flox} (FVB) mouse (also heterozygous for floxed FGFR1 alleles) generated in Task 2. This cross will yield [TRAMP/ *Fgfr1*^{flox} (C57BL/6)]/[FAP(CrePR1)/ *Fgfr1*^{flox} (FVB)] mice. All the experimental mice and controls for the project will be generated from this crossing and progeny screened for the FAP(CrePR1) transgene. In the FAP(CrePR1) transgenic mice there will be approximately 25% progeny that are homozygous for the floxed FGFR1 alleles, which will be the conditional knockout experimental animals (both FGFR1 alleles will be knocked out specifically at reactive stroma sites via Miphepristone-induction). As controls, approximately 50% of the FAP(CrePR1) transgenic mice will be heterozygous for the floxed FGFR1 alleles and 25% will have wild type FGFR1 alleles. All mice will receive Miphepristone and tumors will be evaluated exactly as proposed in the application. FAP should be expressed at the site of reactive stroma formation in the developing TRAMP tumor. Administration of Miphepristone should up regulate Cre recombinase specifically at these sites and the Cre should excise both FGFR1 alleles in the homozygous mice, creating a conditional knockout in tumor reactive stroma. Results will be compared to the TRAMP mice having heterozygous and wild type FGFR1 alleles and hence, functioning FGFR1. When exposed to Cre, a single allele knockout (heterozygous mice for floxed FGFR1 alleles) showed phenotypes similar to wild type controls in the previous studies of Partanen, which focused on developing mid- and hindbrain (11). Hence, we expect the heterozygous and wild type to have similar control phenotypes. The completion of Task 3 will therefore yield data that directly answers the questions of the central hypothesis, as proposed.

Key Research Accomplishments:

Acquisition and verification of BAC clone containing the mouse FAP gene (clone RP23-161B24).
Acquisition of clones containing Miphepristone inducible Cre recombinase (CrePR1) and verification of sequence.
Construction, subcloning and verification of an SV40 Neo selection cassette flanked with Flp recombinase sites upstream of an IRES element and CrePR1.
Acquisition of EL250 cells containing an arabinose inducible *flpe* gene.
Acquisition of the *Fgfr1*^{flox} mice (ICR background) and confirmation of floxed alleles.
Rederivation of the *Fgfr1*^{flox} mice by embryo transfer and initiation of *Fgfr1*^{flox} mice in TMF pathogen free facility.
Acquisition of homozygous TRAMP mice and initiation of colony in TMF facility.
4 generations of crossing *Fgfr1*^{flox} mice into FVB background.
4 generations of crossing *Fgfr1*^{flox} mice into C57BL/6 background.

Reportable Outcomes:

Generation of FlpSV40Flp-IRES-CrePR1 construct.
Rederived colony of pathogen free *Fgfr1*^{flox} mice.
Colony of *Fgfr1*^{flox} mice crossed into FBV background.
Colony of *Fgfr1*^{flox} mice crossed into C57BL/6 background.
Colony of homozygous TRAMP mice.
Generation of an *Fgfr1*^{flox} mouse fibroblast cell line.

Conclusions:

The proposed study was designed to assess the role of FGF receptor 1 signaling in the reactive stroma of an experimental mouse model of prostate cancer. Targeting gene expression specifically to sites of reactive stroma is a key goal of this project. The use of the FAP locus to accomplish this is a novel concept and generation of the FAP(CrePR1) mouse will be a resource for all investigators who study reactive stroma tumor microenvironment.

The completed research to date is fully on track with what we have proposed as the major goals of the study and in the Statement of Work. We have acquired all the materials, clones and lines of cells and mice needed and have rederived them where necessary. We have nearly completed the cloning and construction of the inducible CrePR1 knockin vector. Breeding of mice into the appropriate genetic background is a key aspect of this project and we are nearing completion of this as well. We have encountered no unusual problems and we anticipate no significant problems in completing the steps we have planned out to complete the study as proposed.

This project represents the first time that a transgene will have been expressed specifically in the reactive stroma compartment of a tumor mouse model and represents the first time a gene will be conditionally knocked out in this compartment. Accordingly, this allows us for the first time to be able to study the significance of specific gene expression in the tumor microenvironment. This, in turn, will allow us to address the complex biology of this microenvironment in terms of a targeted therapeutic.

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